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Extracellular Hemoglobins of Hydrothermal Vent Annelids: Structural and Functional Characteristics in Three Alvinellid Species

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Abstract. The polychaete annelids *Alvinella pompejana*, *Alvinella caudata*, and *Paralvinella grasslei* are strictly associated with deep sea hydrothermal vents. Each species possesses an extracellular hemoglobin, Hb, which has been studied and compared to that of a common intertidal polychaete, the lugworm *Arenicola marina*. The four Hbs exhibit very similar quaternary structures and spectral properties, and only small differences appeared in the gross polypeptide compositions after reduction and sodium dodecyl sulfate denaturation of the native molecules. Conversely, by a comparison of the effects of pH (6.6–7.6) and temperature (10–40°C) on their intrinsic O affinities, Bohr factors, cooperativities, and apparent heats of oxygenation, lugworm Hb can be differentiated from that of the alvinellids, and the Hb of *A. pompejana* from that of *A. caudata*. The known biology of the lugworm and a further analysis of the data suggest several hypotheses concerning the *in vivo* O_2 transport function of the alvinellid Hbs, the *in vivo* blood pH value in the two alvinellid species, their respective range of optimal temperature, and their ability to create a differentiated and stable external microenvironment.

Introduction

The known members of the tubicolous polychaete family Alvinellidae are associated only with deep sea hydrothermal vents. In the East Pacific Rise region, the tubes of the closely related species *Alvinella pompejana* and *Alvinella caudata* form honeycomb-like structures covering the external surface of the active vents, where they are frequently associated with the smaller species *Paralvinella grasslei* (Desbruyères and Laubier, 1986). The mixing of the very hot, anoxic vent water (up to 320°C) with the cold, oxygenated deep seawater (2°C) occurs at random. All three alvinellid species are supposed to live on the colder edge of a very sharp thermal gradient, at temperatures as high as 50°C (Desbruyères *et al.*, 1982; Arp and Childress *in* Terwilliger and Terwilliger, 1984). This environment is characterized by high-frequency, unpredictable changes in temperature, pH, oxygen partial pressure, and sulfide concentration (Johnson *et al.*, 1986, 1988).

The alvinellids have well-developed gills (Jouin and Gaill, 1990) and a closed vascular system containing a high molecular weight, extracellular hemoglobin (Hb) dissolved in the blood. These Hbs have rarely been studied, and most of the available data have been obtained by Terwilliger and Terwilliger (1984) on *A. pompejana* Hb. Recently, one of us (A.T.) collected fresh blood directly from living specimens of *A. pompejana*, *A. caudata*, and *P. grasslei*. We describe here the structure and some of the functional properties of the Hbs from these samples. The effects of pH and temperature on the oxygen binding properties of the Hbs were examined at constant inorganic ion concentration and at one atmosphere hydrostatic pressure. For comparison, the same studies were carried out on solutions of the extracellular Hb of a mainly intertidal species, the common lugworm *Arenicola marina*, prepared and stored in the same conditions.

Materials and Methods

Animals

The alvinellids were collected at 2600 m depth in November 1987 during the French-American "Hydronaut"

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expedition on the "13°N" hydrothermal vent site (East Pacific Rise region, Fustec *et al.*, 1987). Large pieces of black or white smokers were plucked off by the external arm of the DSRV *Nautile* and placed in an insulated, non-pressurized container, closed at depth to keep temperature constant as the yellow submarine surfaced. The lugworms were collected on the Penpoull beach near Roscoff, Brittany, France.

Blood collection and Hb solution preparation

Immediately after the alvinellids were recovered on board ship, they were opened dorsally, and the blood, uncontaminated with coelomic fluid, was withdrawn from the main vessels into glass micropipettes and pooled on melting ice. In Roscoff, the same procedure was applied to lugworms kept unfed for 12 to 24 h in local running seawater (temperature 14–16°C). The total blood volumes collected from the alvinellids were around 0.8 ml for *A. pompejana* (10 specimens), 0.7 ml for *A. caudata* (12), and 0.05 ml for *P. grasslei* (3).

The blood was centrifuged at low speed for a few minutes, and the supernatant was divided into two parts. (i) For examination of the Hb molecules by transmission electron microscopy (TEM), a few droplets of the supernatant were diluted 1:200 in a buffer comprising 50 m*M* Bis-tris-propane (BTP; Sigma) and HCl at pH 7.4. The grids were prepared by standard techniques (Valentine *et al.*, 1968), on board ship or in the Roscoff laboratory. (ii) The remaining supernatant was equilibrated against 50 m*M* BTP-seawater/HCl buffer (pH 7.6) by gel filtration on Sephadex G-25, saturated with carbon monoxide, and frozen in liquid nitrogen. In Paris, these samples were thawed, and a metHb-free, HbCO-free, pure HbO_2 solution was prepared using standard techniques (Riggs, 1981).

Spectrophotometric studies

U.V./vis. absorption spectra of the Hbs were obtained at 20°C with a Bausch and Lomb Spectronic 2000 spectrophotometer. The heme concentration of the solutions was determined using a millimolar extinction coefficient $\epsilon = 11.0$ at 540 nm for the cyanmet heme (Van Assendelft, 1970).

Electrophoretic studies

The Hbs were denaturated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of mercaptoethanol (ME). The Hbs and markers (Pharmacia) of low relative molecular mass (M_r) were first heated at 100°C for 5 min in a 2.5% SDS solution, with or without 5% ME. The electrophoresis was then carried out on 10% polyacrylamide slab gels, in 12.5 m*M* Tris/glycine buffer (pH 8.5), with 0.1% SDS.

Functional properties

For studies of the O_2-binding characteristics of the Hbs, aliquots of the pure HbO_2 solutions were equilibrated against 50 m*M* BTP/HCl buffer by gel filtration on Sephadex G-25 (final heme concentration: 40–70 μM). The buffers were adjusted in order to obtain constant pH values: 6.6, 6.9, 7.25, 7.6, whatever the experimental temperature: 10, 20, 30, 40°C. Except for Na^+, which varied between 265 and 305 m*M* depending mostly on the pH value, the inorganic ion concentrations, in m*M*, were also kept constant: $Cl^- = 470$; $SO_4^{2-} = 30$; $Mg^{2+} = 50$; $Ca^{2+} = 10$. These values are similar to those observed in the coelomic fluid of the lugworm (Robertson, 1949). The total osmolarity of the solutions was about 1.06 OsM.

Oxygen-equilibrium curves (OEC) were obtained, with no carbon dioxide in the gas phase and at one atmosphere hydrostatic pressure, by a continuous spectrophotometric method; we used a Hemox Analyser spectrophotometer (TCS, Southampton, Pennsylvania) interfaced with a Hewlett-Packard 85B microcomputer and a Hewlett-Packard ColorPro Graphics plotter. The purified HbO_2 solution was first equilibrated against pure oxygen and then slowly deoxygenated with pure nitrogen or argon. The deoxygenation procedure lasted 60 to 90 min, and the microcomputer was programmed to store up to 300 points of the OEC on tape, each point corresponding to the coupled mean values of 30 and 60 successive measurements of, respectively, oxygen partial pressure (P_{O_2}) and O_2 saturation of the Hb. Negligible quantities of metHb were produced during these experiments, a consequence of the particularly high resistance of lugworm and alvinellid Hbs to oxidation (Toulmond *et al.*, 1988). The P_{O_2} at half saturation of the Hb (P_{50}) was calculated from the experimental values between 40 and 60% O_2 saturation by linear regression analysis, and approximate values of the dissociation constants for the R and T states [respectively, K_R and K_T (Edelstein, 1975)] were estimated graphically from the Hill plot of the OEC. The value of the Hill coefficient [n_{max}, corresponding to the maximum slope of the Hill plot (Imai, 1982)], as well as its position on the saturation axis, were estimated graphically from the calculated first derivative of the Hill plot, the so-called cooperativity curve (Girard *et al.*, 1987).

The Hemox technique gave highly reproducible results, especially in conditions where the Hb affinity is high. The statistical analysis of a preliminary set of 10 OECs, obtained at pH = 7.6 and 20°C on lugworm blood, gave the following mean results (value ± SD): P_{50} (mm Hg) = 1.82 ± 0.04; $n_{50} = 2.36 \pm 0.06$; K_T (mm Hg) = 16.1 ± 1.1; K_R (mm Hg) = 1.21 ± 0.32.

Results

Absorption spectra

Absorption spectra were typical of hemoglobins and quite similar in all the species studied; the position of

Table I

Spectral position in nm of HbO_2 α and β peaks and ratio of absorbance of the α to the β peaks in the four studied species

	Alvinella pompejana	*Alvinella caudata*	*Paralvinella grasslei*	*Arenicola marina*
Peak	574.5	574.2	573.7	573.7
	0.2*	0.3		0.3
Peak	539.8	539.8	539.5	538.8
	0.2	0.2		0.2
A_α/A_β	0.92	0.93	0.96	0.98
	0.01	0.01		0.01
n**	8	8	2	8

* Standard deviation.
** Number of measurements.

Table II

Dimensions in nm of negatively stained Hb molecules as measured on electron micrographs

	Alvinella pompejana	*Alvinella caudata*	*Paralvinella grasslei*	*Arenicola marina*
Maximum diameter	30.4	30.2	29.6	30.0
	0.8*	0.9	1.4	1.2
Side to side width	27.0	27.2	26.5	27.5
	0.8	0.7	1.1	0.7
Height	19.7	19.4	18.9	19.7
	0.9	1.2	1.9	1.0

* Standard deviation; n = 30.

major absorption peaks showed only slight, insignificant differences. Temperature and pH changes had practically no effect on these spectra. In all cases, even in the absence of methemoglobin, the ratio of absorbance of the α peak to the β peak was less than one (Table I).

Molecular structure

In the four species, the electron micrographs of negatively stained native molecules showed the same two-tiered hexagonal structure typical of annelid extracellular Hbs (Fig. 1); the dimensions were practically identical (Table II). FPLC filtration (Fig. 2) on a Superose 6 column (Pharmacia), as well as filtration on a Sepharose 6B column (1.6 × 70 cm), showed that alvinellid and lugworm Hbs have practically the same elution volume and, most probably, similar M_rs.

In the three alvinellid species, denaturation and electrophoresis of the Hbs by SDS-PAGE yielded three major bands corresponding to proteins of M_r about 45,000, 30,000, and 15,000. Two fainter bands were also present corresponding to proteins of M_r *ca.* 28,000 and 22,000 in the genus *Alvinella*, and about 28,000 and 25,000 in the genus *Paralvinella*. By comparison, denaturation of *Arenicola marina* Hb gave four major bands corresponding to M_rs of about 45,000, 32,000, 28,000, and 15,000 (Fig. 3A).

In the four species, reduction by ME and simultaneous denaturation by SDS produced a major band corresponding to polypeptides of M_r between 14,000 and 16,000. Fainter bands corresponded to polypeptides of M_r about 35,000 and 25,000 in the genus *Alvinella*, 30,000 and 28,000 in *Arenicola*, and 28,000 in *Paralvinella* (Fig. 3B).

Oxygen equilibrium studies

Because so little *P. grasslei* blood was available, these studies were carried out only on *A. pompejana*, *A. caudata*,

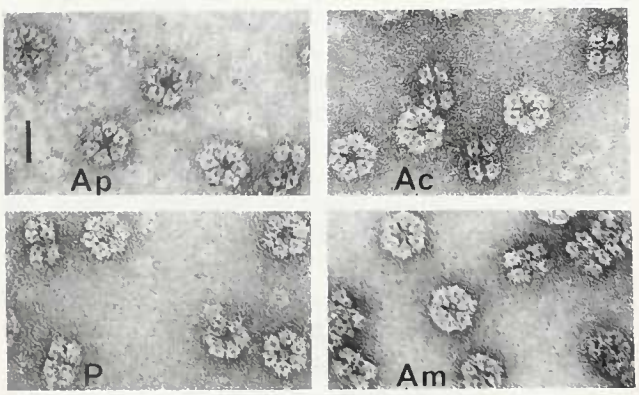

Figure 1. Electron micrographs of native molecules of the four extracellular Hbs, negatively stained with 2% uranyl acetate. Scale bar: 25 nm. (Ap) *Alvinella pompejana*; (Ac) *A. caudata*; (P) *Paralvinella grasslei*; (Am) *Arenicola marina*.

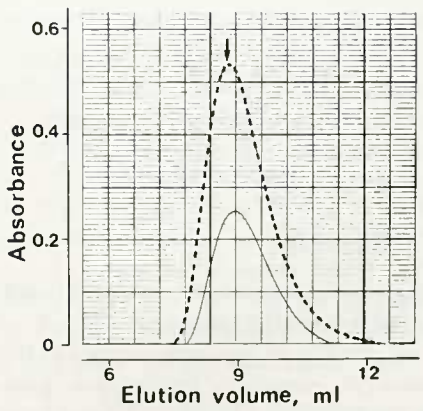

Figure 2. Elution profiles of *Alvinella pompejana* Hb on a Superose 6 column, in Bis-tris-propane/HCl buffer. The arrow indicates the peak position for *Arenicola marina* Hb. Absorbance was measured at 280 nm (solid line) and 410 nm (dashed line).

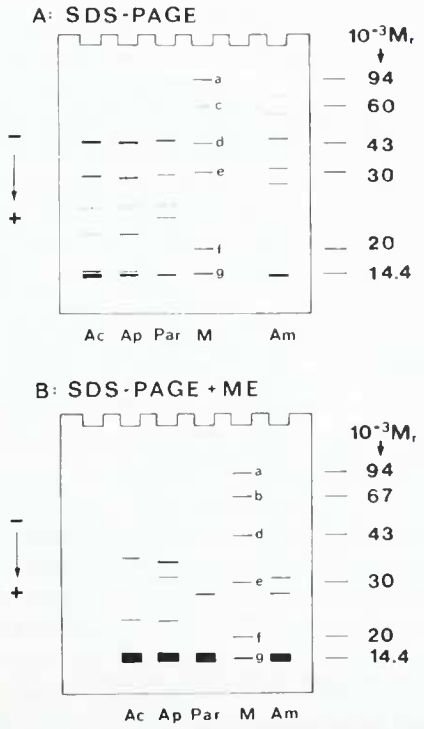

Figure 3. SDS slab gel electrophoresis, 10% polyacrylamide, of the four extracellular Hbs. (A) Before reduction by mercaptoethanol (ME); (B) after reduction by ME. (Ac) *Alvinella caudata*; (Ap) *A. pompejana*; (Par) *Paralvinella grasslei*; (Am) *Arenicola marina*. (M) low molecular mass markers (Pharmacia). (a) Phosphorylase; (b) serum albumin; (c) catalase; (d) ovalbumin; (e) carbonic anhydrase; (f) trypsin inhibitor; (g) lactalbumin.

and *Arenicola marina* Hbs. Figure 4 shows the Hill plot of a typical OEC obtained on *A. pompejana* Hb. *In vitro*, the alvinellid Hbs were characterized by a very high intrinsic O_2 affinity, with P_{50} values very dependent on pH and temperature (Table III). The normal Bohr effect was large, with Bohr factors that may have been lower than -1, and was greatest at low temperature and at low to medium O_2 saturation of the pigment (Table IV). The cooperativity was also high. The Hill coefficient, n_{max}, was in some cases higher than 4 (Fig. 5) and was strongly dependent on pH and temperature, being maximum for pH around 6.6–6.9 (Fig. 6). The apparent heat of oxygenation, ΔH, was also very high, peaking at more than -100 kJ/mol O_2, and strongly pH dependent (Table V).

The two alvinellid Hbs differed significantly with respect to these characteristics: the Bohr effect, cooperativity, and apparent heat of oxygenation were systematically higher in *A. pompejana* than in *A. caudata*. However their Hbs shared particular properties quite different from those of the lugworm. In the same experimental conditions, the lugworm Hb exhibited a lower O_2 affinity, a lesser Bohr effect with maximum values of the Bohr factor at medium to high O_2 saturation, a lower cooperativity with maximum values at rather alkaline pH (about 7.25–7.6), and lower pH-independent values of ΔH.

Discussion

Molecular structure

The alvinellid and lugworm Hbs exhibit the same quaternary structure, and it is typical of annelid extracellular Hbs. For the four molecules, and in the same experimental conditions: (i) FPLC, as well as low-pressure column chromatography, give almost identical elution profiles (Fig. 2) indicating very similar M_rs of about 3.6×10^6; and (ii) the native molecules measured on electron micrographs show only small, nonsignificant differences in dimensions (Table II). These M_rs and dimensions are very close to those recorded in the literature for the Hbs of intertidal polychaetes and terrestrial or aquatic oligochaetes (Vinogradov *et al.*, 1982), and they are very similar to those obtained in a recent small angle X-ray scattering study of lugworm Hb (El Idrissi Slitine *et al.*, 1990).

From these observations, we consider that the decrease in hydrostatic pressure experienced by the alvinellid blood during the submarine's rise to the surface (about 260 atmospheres) had little or no effect on the shape, size, structure, and, consequently, on the functional properties of alvinellid Hbs. Three observations support this opinion: (i) the electron micrographs show that both alvinellid and lugworm Hbs dissociate into more or less spheroidal particles, probably corresponding to twelfths of the native molecules; but the proportion of these particles is nearly

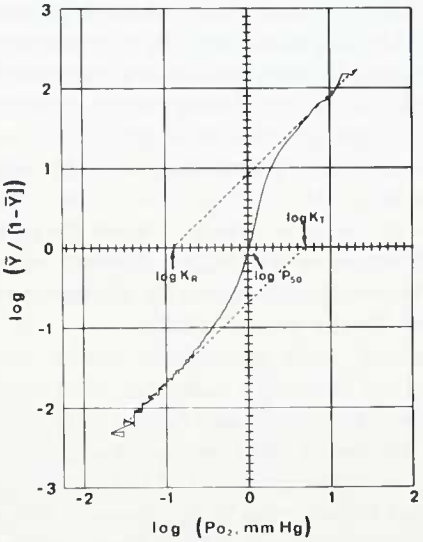

Figure 4. Hill plot of a typical oxygen equilibrium curve of *A. pompejana* extracellular Hb. Log K_R and log K_T were graphically estimated at the intersection of the log P_{O_2} axis by straight lines (slope = 1) drawn asymptotic to the Hill plot at extreme high and low O_2 saturation values, respectively. pH 6.90; 20°C; heme concentration: 70 μM.

Table III

P_{50} in mm Hg as a function of pH, 6.6 to 7.6, and temperature, 10 to 40°C. Each value was obtained from one, rarely two, O_2-binding curves

		6.6	6.9	7.25	7.6
Alvinella pompejana	10°C	0.5	0.2	0.1	ND*
	20°C	1.9	1.0	0.3	0.2
	30°C	3.9	1.8	0.9	0.5
	40°C	8.1	4.4	2.9	2.2
Alvinella caudata	10°C	0.5	0.4	0.2	0.1
	20°C	1.8	1.0	0.4	0.3
	30°C	3.8	2.0	1.1	0.8
	40°C	6.6	4.4	2.5	2.4
Arenicola marina	10°C	5.7	4.2	2.8	1.4
	20°C	9.5	6.5	3.7	2.1
	30°C	13.9	9.1	5.4	3.3
	40°C	15.6	10.5	5.8	4.3

* Not done.

Table IV

Effect of temperature, 10 to 40°C, on the mean Bohr factor calculated between pH 6.6 and 7.6 for almost completely deoxygenated ($\phi T = \Delta \log K_T/\Delta pH$), half-oxygenated ($\phi P_{50} = \Delta \log P_{50}/\Delta pH$), and almost completely oxygenated ($\phi R = \Delta \log K_R/\Delta pH$) Hbs

Bohr coefficient		ϕT	ϕP_{50}	ϕR
Alvinella pompejana	10°C	−1.60	−1.17	−0.05
	20°C	−1.21	−1.18	−0.24
	30°C	−1.20	−0.89	+0.02
	40°C	−0.86	−0.56	−0.08
Alvinella caudata	10°C	−0.35	−0.76	−0.15
	20°C	−0.93	−0.90	−0.30
	30°C	−0.92	−0.68	−0.03
	40°C	−0.78	−0.47	−0.03
Arenicola marina	10°C	−0.18	−0.62	−0.44
	20°C	−0.34	−0.66	−0.43
	30°C	−0.34	−0.64	−0.38
	40°C	−0.44	−0.58	−0.38

the same for all species, indicating either their normal presence in the blood *in vivo*, or, most probably, their unavoidable formation during the preparation of the grids for the TEM study. (ii) A recent study has shown that hydrostatic pressure dissociates annelid extracellular Hbs significantly only when it is *increased* to more than 1000 atmospheres (Silva *et al.*, 1989); a *decrease* in hydrostatic pressure, from about 260 to 1 atmosphere, would be unlikely to substantially affect the quaternary structure of these Hbs. (iii) Preliminary experiments have shown that, during a progressive increase of the hydrostatic pressure up to 1500 atmospheres, followed by a progressive decrease back to one atmosphere, the absorbance spectrum of half-oxygenated lugworm Hb is not appreciably modified, indicating that no change occurs in either the O_2 saturation or the O_2 affinity of the Hb (Hui Bon Hoa and Toulmond, unpub.). Nevertheless, we must keep in mind that, on the basis of data obtained *in vitro* at 1 atmosphere hydrostatic pressure, we compare below the properties of Hbs that function *in vivo* at two different values of hydrostatic pressure (1 atmosphere for the lugworm Hb, 260 atmospheres for the alvinellid Hbs).

We obtained some information about the detailed structure of the native Hb molecules. SDS denaturation confirms that these molecules belong to the annelid extracellular Hb family, with only small variations around the general type (Vinogradov, 1980). However, small differences exist between the electrophoretic patterns of *A. pompejana* and *A. caudata* Hbs. These differences, together with those concerning the functional properties discussed below, confirm the distinct taxonomic status of these two recently separated species (Autem *et al.*, 1985; Desbruyères and Laubier, 1986).

Physicochemical and functional properties

Alvinellid Hbs can be easily distinguished from lugworm Hb in that there are notable differences of intrinsic O_2 affinity, Bohr effect, cooperativity, and apparent heat of oxygenation. In a detailed examination of these properties, the Hb of *A. pompejana* can be distinguished from that of *A. caudata*. Can these differences be correlated with what is known of the specific characteristics of the animals and their environment?

The high intrinsic O_2 affinity of *A. pompejana* Hb has already been reported by Terwilliger and Terwilliger (1984). We confirm here that the O_2 affinity of both *A. pompejana* and *A. caudata* Hbs is very high whatever the

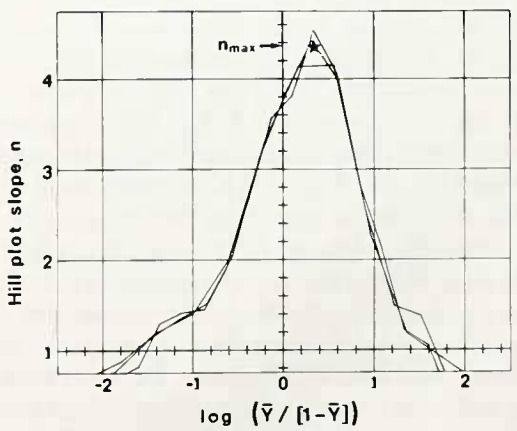

Figure 5. Three different calculations of the first derivative of the Hill plot of Figure 4, showing the variations of the Hill plot slope, n, as a function of log ($\bar{Y}/(1 - \bar{Y})$). n_{max}: the graphically estimated value of the Hill coefficient.

experimental conditions (Table III), 2 to 10 times higher than that of the lugworm which has a Hb affinity for O_2 that is already quite high (for a comparison with other annelid Hbs, see Weber, 1980). In the extreme conditions of low temperature (10°C) and high pH (7.6), the O_2 affinity of *A. pompejana* Hb was so high (P_{50} lower than 0.1 mm Hg, with 1 mm Hg = 133.3 Pa) that it could not be measured with the Hemox technique. Hbs with high O_2 affinities are generally considered very adaptive in species lacking an efficient, specialized respiratory organ (see Weber, 1978). But alvinellid gills are characterized by the highest specific surface areas yet measured in polychaetes, low diffusion distances between the external seawater and the blood, and a branchial circulatory system with a complexity comparable to that of the fish gill (Jouin and Gaill, 1990).

Hbs with high O_2 affinity can also be advantageous to species living in a poorly oxygenated environment (Weber, 1980). But what do the alvinellids actually breathe? According to Desbruyères *et al.* (1982) and Arp and Childress (*in* Terwilliger and Terwilliger, 1984), a mild to warm (up to 50°C), hypoxic water: *i.e.*, a mixture of the very hot, anoxic vent water and the cold, oxygenated local bottom seawater. But the oxygen concentration of this water mix has never been directly measured *in situ*, and the only direct evidence for low O_2 concentrations inside and outside hydrothermal vent community come from the Rose Garden vent field in the Galapagos Rift (Johnson *et al.*, 1986), where the O_2 content is always below 1/3 of the saturation at one atmosphere hydrostatic pressure. However, alvinellids have never been seen at the Rose Garden site, and the conditions there are quite different from those at the 13°N site. The hypothesis that alvinellids breathe hypoxic water must be considered, but is as yet not really supported.

The high O_2 affinity of alvinellid Hbs is modulated by the very large Bohr effect we found. The magnitude of the Bohr effect is extremely dependent on the oxygenation

Table V

Heat of oxygenation, $\Delta H = 2.303R\Delta \log P_{50}/\Delta(T^{-1})$, kJ/mol O_2. Mean values calculated between 10 and 40°C, for 4 pH values, 6.6 to 7.6

	6.6	6.9	7.25	7.6
Alvinella pompejana	−66 −0.976*	−76 −0.990	−89 −0.998	−102 −0.999
Alvinella caudata	−57 −0.990	−63 −0.998	−69 −0.999	−76 −0.995
Arenicola marina	−23 −0.972	−23 −0.977	−24 −0.987	−24 −0.993

* Correlation coefficent.

of the Hb molecule (Table IV): it is maximum when the molecule, almost fully deoxygenated, is in the so-called T-state (S_{O_2} *ca* 0%); minimum or null when the molecule, almost completely oxygenated, is in the R-state (S_{O_2} *ca* 100%); and intermediate when the molecule is half-oxygenated at P_{50}. These S_{O_2}-dependent Bohr-effect variations must greatly facilitate the O_2 unloading of the pigment at the tissue level, an advantage in view of the very high intrinsic O_2 affinity of alvinellid Hbs. The Bohr effect of the lugworm Hb is not as strong, and the maximum values of the Bohr factor occur when the Hb is half or nearly completely oxygenated, a property that Weber (1981) sees as favoring the O_2 loading of the pigment at the gill.

The oxygen transport efficiency of a respiratory pigment also depends on its cooperativity because, *in vivo*, a maximal cooperativity allows a maximal O_2 loading or unloading of the molecule for a corresponding minimal change of blood P_{O_2}. In alvinellid as well as in *Arenicola* Hbs, the O_2-binding process is highly cooperative, with n_{max} values that can be above 4 in *A. pompejana*. The value of n_{max} varies much more with temperature and pH in alvinellid than in lugworm Hbs (Fig. 6). In *Arenicola*,

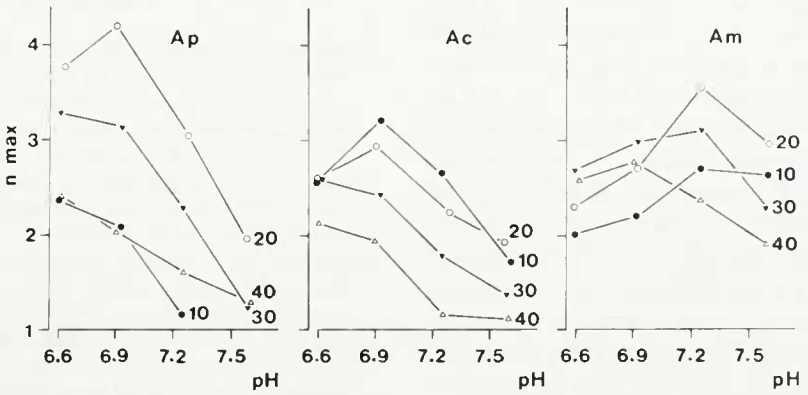

Figure 6. Variations of the Hill coefficient, n_{max}, as a function of pH at 10, 20, 30 and 40°C. Other experimental conditions: see text. (Ap) *Alvinella pompejana*; (Ac) *A. caudata*; (Am) *Arenicola marina*

which normally lives in cold to temperate waters (Wells, 1963), the physiological blood pH is 7.25 and 7.58 in animals acclimated at 26 and 5°C, respectively (Toulmond, 1977). The cooperativity of lugworm Hb is maximum between pH 7.25 and 7.6, and for temperatures between 10 and 30°C (Fig. 6). If the maximum cooperativity of the respiratory pigment is correlated with the physiological pH value in *Alvinella*, as it is in *Arenicola*, then the physiological range of blood pH in *Alvinella* is probably 6.6–6.9 and, in this pH range, the maximum cooperativity is obtained at 10–20°C in *A. caudata*, and at 20–30°C in *A. pompejana*. This blood pH range is unusually low for annelids and only direct measurements of blood pH could validate our interpretation of the data. But it is noteworthy that in the vestimentiferan worm *Riftia pachyptila*, another hydrothermal vent dweller living in sulfide-rich water, similar slightly acidotic pH values have been measured *in vivo* (Childress *et al.*, 1984).

Our observations suggest two sets of hypotheses: (i) the two alvinellid species form sympatric mixed populations on the same white or black smokers, but the external microenvironment is probably slightly colder for *A. caudata*, at 10 to 20°C, than for *A. pompejana*, at 20 to 30°C. These temperatures are well below the maximum temperature, 50°C, that the animals are supposed to withstand *in situ*. Our findings would corroborate Terwilliger and Terwilliger's (1984) observation that *A. pompejana* Hb is unstable at such a high, and probably nonphysiological, temperature. (ii) At those pH and temperature values, the *in vitro* intrinsic O_2 affinities of the Hbs are finally not so high, with P_{50} values ranging from 1.0 to 3.9 mm Hg in *A. pompejana* and from 0.4 to 1.8 mm Hg in *A. caudata* (Table III). The characteristics of the Bohr effect in these animals make such values quite compatible with an *in vivo* O_2 transport function for the Hbs. The additional hypothesis of Terwilliger and Terwilliger (1984), that the greater the depth, the greater the drop in the Hb O_2 affinity caused by hydrostatic pressure, then becomes unnecessary.

The lugworm and alvinellid Hbs differ by another characteristic. In the lugworm, the apparent heat of oxygenation, ΔH, is quite low, about −25 kJ/mol, and pH-independent. By contrast, in alvinellid Hbs, ΔH is strongly pH-dependent and is about three times higher, at pH 6.6–6.9, than in the lugworm Hb (Table V). These high ΔH values explain the important effects of a temperature change on the intrinsic O_2 affinity, the Bohr effect, and the cooperativity. A general inverse relationship can be established between the value of ΔH and the range of temperatures at which a given respiratory pigment has to function *in vivo*: the larger the temperature range, the lower the value of ΔH (see Toulmond, 1985, for examples). Since ΔH is higher for alvinellid than for lugworm Hbs, the alvinellids probably live in an environment better temperature-regulated than that of the intertidal lugworm. This conclusion might seem to be inconsistent with the supposed extreme environmental variability around the hydrothermal vents, but annelids, and especially those living in elaborate tubes or galleries, are capable of creating their own regulated microenvironment (Toulmond, 1990). But as for O_2 concentrations, *in situ* direct measurements of the temperature microdistributions inside and outside the alvinellid tubes are needed.

In conclusion, although the alvinellid Hbs are structurally very similar to those of annelids living in more ordinary habitats, these Hbs clearly exhibit some distinct functional properties that are most probably directly related to the characteristics of the hydrothermal vent environment. Their properties suggest that the alvinellid Hbs function as O_2 carriers at slightly acidic blood pH values and at fairly constant temperatures, not exceeding 20°C for *A. caudata* and 30°C for *A. pompejana*. This could indicate that both species can create a differentiated and stable external microenvironment.

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